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(57) Abstract

The present invention provides an isolated and purified protein that associates with the cytoplasmic domain of the p80 form of the tumor necrosis factor receptor, having a molecular weight of 59 kDa on SDS-PAGE, is a phosphoprotein, and does not bind to the p60 form of the tumor necrosis factor receptor. Also provided is an isolated and purified protein kinase that associates with the cytoplasmic domain of the p80 form of the tumor necrosis factor receptor, said kinase phosphorylates both the p80 and p60 forms of the tumor necrosis factor receptor and phosphorylates a 59 kDa molecular weight protein associated with the cytoplasmic domain of the p80 form of the tumor necrosis factor receptor. Also provided are various methods of reducing the biological effects of tumor necrosis factor.

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# TUMOR NECROSIS FACTOR RECEPTOR-II-ASSOCIATED PROTEIN KINASE AND METHODS FOR ITS USE

## BACKGROUND OF THE INVENTION

### Field of the Invention

The present invention relates generally to the fields of cytokine physiology and protein chemistry. More specifically, the present invention relates to a novel serine-threonine protein kinase associated with the p80 form of the tumor necrosis factor receptor and various methods involving manipulating this protein kinase.

### Description of the Related Art

Tumor necrosis factor (TNF), a homotrimer of 17 kDa subunits, is a cytokine produced mainly by activated macrophages and several other cell types. TNF is pleiotropic, induces cytotoxicity, cachexia, and septic shock, shows anti-viral, anti-inflammatory, and immunoregulatory activities, and stimulates the growth of certain cells. Two different receptors, termed p60 (also known as p55 or TNFR-I) and p80 (also known as p75 or TNFR-II), bind TNF with high affinity. The extracellular domain of both the p60 and p80 forms of the TNF receptor share 28% sequence identity with each other and other members of the TNF/nerve growth factor (NGF) receptor family. Little homology exists between the cytoplasmic domains of p60 and p80 forms of the TNF receptor.

Intracellular events linking the activation of TNF receptors to specific cellular responses are unclear. TNF activates the transcriptional complex nuclear factor kappa B (NFkB) within minutes, transmitting a signal from the receptor to the nucleus. Additionally, a TNF signaling pathway involving the breakdown of sphingomyelin to ceramide and stimulation of a ceramide-activated kinase have been described. TNF augments the phosphorylation state of several proteins such as the small heat shock protein (hsp 27), the eukaryotic initiation factor 4E, the inhibitory subunit of NF-$\kappa$B (I$\kappa$B-$\alpha$), and the epidermal growth

factor receptor. Various protein kinase activities have been demonstrated to be rapidly and transiently activated upon TNF treatment.

The functional role of the cytoplasmic domain of p60 for signaling the cytotoxic response to TNF has been reported. Brakebusch et al. expressed a series of truncated human p60 receptors in murine L929 cells and observed that deletion of 50% of the cytoplasmic domain resulted in the loss of the TNF cytotoxic effect but not shedding of the receptor. Similarly, Tartaglia et al. demonstrated that expression of human p60 lacking most of the cytoplasmic domain rendered L929 cells defective in TNF responses. Additionally, Tartaglia et al. showed that a region within the cytoplasmic domain of p60 termed the "death domain" (residues 324-426) was necessary for generation of the TNF cytotoxic signal in mouse L929 cells. Thus, the cytoplasmic domain may contain structural information necessary for interaction with intracellular components required for TNF signaling.

Receptors that lack kinase activity transmit their signals through recruitment of specific kinases by their cytoplasmic domains. However, there has been no evidence of any proteins in association with the cytoplasmic domain of either the p60 or the p80 forms of the tumor necrosis factor receptor. Manipulation of such a protein would provide an avenue for regulation of TNF's biological activities.

In spite of the conserved features of the extracellular domains of the p60 and p80 forms of the tumor necrosis factor receptor, it has been difficult to identify common motifs in their intracellular regions. Like other members of the TNF/NGF receptor family, the p60 and p80 forms of the TNF receptor do not contain consensus sequences characteristic of tyrosine or serine/threonine kinases, or any other signal transduction motifs. However, ligand binding to the TNF receptor activates a wide variety of putative second-messenger events, including a rapid increase in protein phosphorylation. It is unclear which of these processes form the link between ligand binding at the

cell-surface and the profound effects that TNF has upon cell function.

Efforts to identify receptor domains critical for cellular signaling have relied on mutational analysis. The deletion analysis reported by Brakebusch et al. indicated that truncation of at least half of the cytoplasmic domain of p60 abolished the ability of TNF to signal for cytotoxicity. Additionally, a mutant receptor lacking most of its cytoplasmic domain interfered with the endogenous wild-type receptor, suggesting that receptor clustering is necessary for signal transmission. Similarly, Tartaglia et al. demonstrated that the expression of a truncated human p60 receptor in mouse cells suppressed the signaling of the endogenous mouse TNF receptors in response to the ligand. Interestingly, the death domain shares weak homology with a region found in the cytoplasmic domain of the Fas antigen that is necessary for apoptotic signal transduction.

The prior art is deficient in the lack of effective means of inhibiting the various biological activities of tumor necrosis factor. The present invention fulfills this longstanding need and desire in the art.

## SUMMARY OF THE INVENTION

The present invention identify proteins that associate with the cytoplasmic domain of the p80 form of the TNF receptor. The present invention provides evidence of physical and functional association of a novel protein kinase that interacts with the cytoplasmic domain of the p80 form of the TNF receptor.

In one embodiment of the present invention, there is provided an isolated and purified protein that associates with the cytoplasmic domain of the p80 form of the tumor necrosis factor receptor, having a molecular weight of 59 kDa on SDS-PAGE, is a phosphoprotein, and does not bind to the p60 form of the tumor necrosis factor receptor. The protein of the present invention is termed herein Tumor Necrosis Factor Receptor-II Associated Protein (p80TRAP).

In another embodiment of the present invention, there is provided an isolated and purified protein kinase that

associates with the cytoplasmic domain of the p80 form of the tumor necrosis factor receptor, said kinase phosphorylates both the p80 and p60 forms of the tumor necrosis factor receptor and phosphorylates a 59 kDa molecular weight protein associated with the cytoplasmic domain of the p80 form of the tumor necrosis factor receptor. The kinase of the present invention is termed herein Tumor Necrosis Factor Receptor-II Associated Kinase (p80TRAK).

In yet another embodiment of the present invention, there is provided a pharmaceutical composition comprising an competitive inhibitor of the kinase or p80TRAK of the present invention and a pharmaceutically acceptable carrier.

In yet another embodiment of the present invention, there is provided a method of treating a pathophysiological state characterized by an undesirable physiological level of tumor necrosis factor comprising the step of administering a pharmacologically effective dose of the pharmaceutical composition of claim 5 to a human.

In other embodiments of the present invention, there are provided various methods of decreasing the biological effects of tumor necrosis factor and reducing the cytotoxic effects of tumor necrosis factor.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

**BRIEF DESCRIPTION OF THE DRAWINGS**

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

The following abbreviations are used in the instant specification: TNF: tumor necrosis factor; NGF: nerve growth factor; NF-κB: nuclear factor kappa B; IκB-α: inhibitory subunit of NF-κB; FBS: fetal bovine serum; PCR: polymerase chain reaction; MBP: myelin basic protein; GST: glutathione-S-transferase; CD: cytoplasmic domain; PMSF: phenylmethylsulfonyl fluoride; SDS: sodium dodecyl sulfate; PAGE: polyacrylamide gel electrophoresis; PVDF: polyvinylidene difluoride; TLC: thin layer chromatography; p80TRAK: tumor necrosis factor receptor-associated kinase; and p80TRAP: tumor necrosis factor receptor-associated protein.

Figure 1 shows the association of proteins with GST-p80CD from $^{35}$S-labeled (left) and $^{32}$P-labeled (right) U-937 cells. Left, U-937 cells (4 x $10^6$/ml) were labeled for 4 hours at 37°C with a mixture of $^{35}$S methionine/cysteine (50 μCi/ml) in 30 ml of methionine/cysteine-free RPMI-1640 medium supplemented with 10% dialyzed FBS. Cells were collected, washed 3x with RPMI-1640, adjusted to 1.5 x $10^6$/ml, and incubated at 37°C for 1 hour. Cells were treated with 5 nM TNF for 5 minutes, washed 2x with cold phosphate-buffered saline, lysed, and cell extracts allowed to bind to GST and GST-p80CD. Adsorbed proteins were subjected to 7.5% SDS-PAGE, and the dried gel was analyzed by a Phosphorimager. Molecular masses in kDa are as indicated; the arrow indicates the position of the 59-kDa protein. Right, U-937 cells (4 x $10^6$/ml) were labeled for 2 hours with carrier-free [$^{32}$P]orthophosphate (500 μCi/ml) in 30 ml of phosphate-free RPMI-1640. Cells were washed 3x with fresh medium, treated with 5 nM TNF for 5 minutes.

Figure 2 shows the kinase activity associates and phosphorylates GST-p80CD. Cells (2 x $10^6$) were lysed and *in vitro* binding to 5 μg of either GST or GST-p80CD was determined. For *in vitro* kinase reactions, samples were incubated in the absence (-) or presence (+) of Mg-ATP for 10 minutes at 37°C. In the mock reaction, no cell extract was added. Proteins were subjected to 10% SDS-PAGE and visualized by staining with Coomassie Blue. The dried gel was analyzed by a Phosphorimager.

Figure 3 shows the characterization of GST-p80CD-associated kinase activity. Binding of U-937 cell extracts (2 x $10^6$) to GST-p80CD was determined. Figure 3A shows that the *in vitro* kinase assays were performed on proteins adsorbed to GST-p80CD with the indicated cation concentrations for 10 minutes at 37°C. Figure 3B shows that after *in vitro* kinase assay, phosphoamino acid analysis of GST-p80CD was performed. The migrations of phosphoamino acid standards are shown. Figure 3C shows that the *in vitro* kinase assays were performed with 10 mM $MgCl_2$ (left) or 10 mM $MgCl_2$ or $MnCl_2$ (right) and the corresponding substrates for 10 minutes at 37°C and subjected to 12% (left) or 7% (right) SDS-PAGE. Arrows indicate positions of proteins after staining with Coomassie Blue. Shown are phosphorimages of the dried gels.

Figure 4 shows that the p80 receptor-associated kinase activity is increased by TNF treatment. Serum-starved U-937 cells (2 x $10^6$) were incubated with 5 nM TNF for the indicated time. *In vitro* binding to GST-p80CD and kinase assays in the presence of 10 mM $MgCl_2$ and MBP were performed as described below. Proteins were subjected to 12% SDS-PAGE; a phosphorimage of the dried gel is shown.

Figure 5 shows a SDS-PAGE of purified GST-p80CD. Molecular mass standards of the indicated size, expressed in kDa, and approximately 10 $\mu g$ of purified GST-p80CD were subjected to 7.5% SDS-PAGE. The gel was stained with Coomassie Blue.

Figure 6 shows the results of *in vitro* kinase assays of 1 M and 2 M NaCl elutions of p80-TRAK with casein, MBP, and GST-p80CD as substrates. Standard kinase assays were performed with a 5 $\mu l$ portion of the 1 M and 2 M elutions with the indicated substrate. Proteins were subjected to 12% SDS-PAGE, the protein bands were visualized by staining with Coomassie Blue and the dried gel was analyzed by a PhosphorImager.

Figure 7 shows a phosphoamino acid analysis of pp59 or p80TRAP phosphorylated *in vitro* and *in vivo*. *In vivo* and *in vitro* phosphorylation of pp59 or p80TRAP was performed as described above, except that the SDS-PAGE gel was transferred to

PVDF by electrophoresis, p80TRAP was excised from the filter and subjected to phosphoamino acid analysis as described above.

**DETAILED DESCRIPTION OF THE INVENTION**

The present invention is directed to a composition of matter comprising an isolated and purified protein that associates with the cytoplasmic domain of the p80 form of the tumor necrosis factor receptor, having a molecular weight of 59 kDa as determined by SDS-PAGE, is a phosphoprotein, and does not bind to the p60 form of the tumor necrosis factor receptor. Moreover, this phosphoprotein of the present invention is phosphorylated at serine and threonine residues and exhibits optimal phosphorylation in the presence of Mn††, and to a lesser extent, Mg††.

The present invention is also directed to an isolated and purified protein kinase that associates with the cytoplasmic domain of the p80 form of the tumor necrosis factor receptor, said kinase phosphorylates both the p80 and p60 forms of the tumor necrosis factor receptor and phosphorylates the 59 kDa molecular weight protein of the present invention that is associated with the cytoplasmic domain of the p80 form of the tumor necrosis factor receptor.

It is specifically contemplated that pharmaceutical compositions may be prepared using the novel protein of the present invention. In such a case, the pharmaceutical composition comprises the novel protein of the present invention and a pharmaceutically acceptable carrier. A person having ordinary skill in this art would readily be able to determine, without undue experimentation, the appropriate dosages and routes of administration of the novel protein of the present invention. Accordingly, the present invention also comprises a pharmaceutical composition comprising an competitive inhibitor of the kinase of the present invention and a pharmaceutically acceptable carrier.

The present invention also comprises a method of decreasing the biological effects of tumor necrosis factor comprising the step of inhibiting the phosphorylation of the p80TRAP protein of the present invention. Accordingly, the

p80TRAP protein of the present invention is inhibited by administering a pharmacologically effective dose of the pharmaceutical composition of the present invention. Further, the present invention includes a method of decreasing the biological effects of tumor necrosis factor comprising the step of inhibiting the p80TRAK kinase of claim 4 and a method of reducing the cytotoxic effects of tumor necrosis factor comprising the step of inhibiting the p80TRAK kinase of claim 4.

In another embodiment, the present invention is directed to a method of treating a pathophysiological state characterized by an undesirable physiological level of tumor necrosis factor comprising the step of administering a pharmacologically effective dose of the pharmaceutical composition of the present invention to a human.

Generally, the pathophysiological state or conditions treated by the methods of the present invention is any state in which inhibition of the biological effects of tumor necrosis factor is desirable. Accordingly, the pathophysiological states or conditions treated may be ones in which the physiological concentrations of TNF and consequently, the biological effects, are undesirably high. Alternatively, the methods of the present invention may be used to treat pathophysiological state where the level of TNF is "normal" but a reduction or inhibition of the physiological effects of TNF is therapeutically desirable. It is also contemplated that the methods of the present invention may be useful in treating "normal" states or conditions where a reduction or inhibition of the physiological effects of TNF is therapeutically desirable.

Thus, the methods of the present invention may be used to treat such conditions as neoplastic diseases, the human immunodeficiency disease, sepsis, cachexia, graft vs host disease, autoimmune diseases, cerebral malaria and capillary leak syndrome. Representative examples of neoplastic diseases include leukemia, ovarian carcinoma, renal cell carcinoma, breast adenocarcinoma and glioblastoma. Representative examples of autoimmune diseases include systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis.

In another embodiment, the present invention is directed to a method of treating a neuro-oncologic state, comprising administering to a human a pharmacologically effective dose of the pharmaceutical composition of the present invention. Preferably, the neuro-oncologic state is glioblastoma, an astrocytoma or a meningioma.

Also provided by the present invention is a method of treating renal cancer comprising administering to a human a pharmacologically effective dose of the pharmaceutical composition of the present invention.

The level of ordinary skill of the average scientist in the area of molecular cell biology has increased substantially in recent years. A person having ordinary skill in this art would readily be able to sequence the phosphoprotein (p80TRAP) and protein kinase (p80TRAP) of the present invention, given the teachings herein.

With knowledge of the teachings of the present invention, a person having ordinary skill in this art would readily be able to prepare specific competitive inhibitors of the protein kinase of the present invention. That is, a person having ordinary skill in this area of research would be readily able to localize the phosphorylation site on the substrate phosphorylated by the kinase and subsequently use this knowledge to develop competitive inhibitors of the kinase.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

## EXAMPLE 1

### Materials

Human TNF ($5 \times 10^7$ units/mg) was a gift of Genentech (South San Francisco, CA). All reagents were reagent grade or higher. The histiocytic lymphoma cell line U-937 (CRL 1593) was obtained from the ATCC. Cells were grown in RPMI-1640 medium supplemented with 10% FBS and 100 $\mu$g/ml streptomycin at 37°C in a 5% $CO_2$ incubator. *Escherichia coli* strain BL21 was a gift of Dr. V. W. Rodwell (Purdue University, W. Lafayette, IN). *E. coli* strain NM522 and plasmid pGEX-2TH were gifts of Dr. H. Saya

(M. D. Anderson Cancer Center, Houston, TX). The plasmid containing the entire coding sequence of p80 (pCMVXVBpL4-p80) was a gift from Dr. T. Kohno (Synergen, Boulder, CO). Construction of the plasmid encoding GST-p60CDΔ1 was as follows:

Construction of Glutathione-S-Transferase (GST) Expression Vectors

DNA manipulations were carried out as described by Sambrook et al., (1989) Molecular cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Polymerase chain reaction (PCR) and specific 5' and 3' primers with unique restriction sites were used to amplify the cytoplasmic domain of p60 from pCMVXVBpL4-p60 for insertion into the GST fusion vectors. The PCR primers were:

5'-CTAAGAGAATTCGCTACCAACGGTGGAAGTCC-3' and

5'-GACGTACTCGAGTCATCTGAGAAGACT-3'

and were used to amplify a 671-bp fragment that encodes residues Y207 to R426 of p60. The PCR fragment was digested with *Eco*RI and *Xho*I and ligated into *Eco*RI/*Xho*I-digested pGEX-KG to give rise to pGEX-KG-p60CD. The pGEX-KG-p60CD was digested with *Eco*RI and partially digested with *Hind*III, and both the 700-bp (*Eco*RI/*Hind*III fragment) and the 570-bp (*Hind*III/*Hind*III due to an internal *Hind*III site in the p60 gene) fragments were isolated. The 700-bp *Eco*RI/*Hind*III fragment was inserted into *Eco*RI/*Hind*III-digested pGEX-2TH and termed pGEX-2TH-p60CD. In order to place the p60CD coding sequence in frame with GST, pGEX-2TH-p60CD was further digested with *Bam*HI, filled in with Klenow, and relegated to give rise to pGEX-2THΔB-p60CD. Additionally, the 570-bp *Hind*III/*Hind*III fragment was inserted into *Hind*III-digested pGEX-2THΔB to give pGEX-2THΔB-p60CDΔ1.

**EXAMPLE 2**

Construction, Expression, and Purification of GST Fusion Protein

All subsequent DNA manipulations were carried out as described by Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. PCR and the primers

5'-CTAAGAGGATCCAAAAAGAAGCCCTTGTGCCTG-3' and

5'-TCTTAGAAGCTTTTAACTGGGCTTCATCCCAGC-3'

with unique restriction sites were used to amplify a 546-bp fragment from pCMVXVBpL4-p80 for insertion into pGEX-2TH. This construct, pGEX-2TH-p80CD, expresses a fusion protein (GST-p80CD) with the p80 cytoplasmic domain (K266-S439) linked to GST. Expression of GST-p80CD in BL21 cells and purification were carried out as follows: BL21 cells harboring the expression plasmid were induced with 0.5 mM IPTG at 37°C for one hour. Cells were collected by centrifugation and lysed in Buffer A (20 mM Tris, pH 8.0, 200 mM NaCl, 10% glycerol, 0.5% NP-40, 1 mM PMSF, 2 μg/ml aprotinin, μg/ml leupeptin, 0.1% 2-mercaptoethanol) containing 5 mg lysozyme and briefly sonicated. The lysate was cleared by centrifugation at 30,000 rpm and the supernatant passed once through a 1.2 ml column of 50% (v/v) glutathione-agarose. The column was subsequently washed with 20 ml Buffer A, 10 ml of 1 M NaCl in Buffer A and 20 ml Buffer A.

The protein was stored at 4°C on glutathione-agarose beads as a 50% slurry in Buffer A (20 mM Tris, pH 8.0, 200 mM NaCl, 10% glycerol, 0.5% NP-40, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 0.1% 2-mercaptoethanol. The amount of fusion protein was estimated by Coomassie Blue staining of SDS-PAGE.

## EXAMPLE 3

### *In Vitro* Binding of GST Fusion Protein to Cell Extracts

U-937 cells were treated as described above and lysed in 1 ml of lysis buffer (20 mM Tris, pH 7.7, 0.5% NP-40, 200 mM NaCl, 50 mM NaF, 0.2 mM sodium orthovanadate, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 0.1% 2-mercaptoethanol) on ice for 10 minutes followed by 10 minutes of centrifugation. The supernatant was precleared with 25 μg GST and 50 μl 50% (v/v) glutathione-agarose for 2-12 hours at 4°C. The precleared supernatant was mixed with approximately 5-10 μg GST-p80CD that is bound to glutathione-agarose for 2 hours at 4°C. The beads were collected by centrifugation and washed extensively with lysis buffer (4x 500 μl) and with kinase buffer (3x 500 μl: 20 mM HEPES, pH 7.4, 10 mM NaF, 0.2 mM sodium orthovanadate, and 0.1% 2-mercaptoethanol). The pellets were then used for *in vitro* kinase assays.

## EXAMPLE 4

*In Vitro* Kinase Assays

Standard kinase assays were carried out for 10 minutes at 37°C in 50 $\mu$l containing 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM NaF, 0.1 mM sodium orthovanadate, and 10 $\mu$Ci [$\gamma$-$^{32}$P]ATP or as described. Reactions were stopped with 15 $\mu$l SDS-sample buffer, boiled for 5 minutes and then subjected to SDS-PAGE. Protein bands were visualized by staining with Coomassie Blue and the dried gels were analyzed by a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Labeled protein bands were quantitated by using ImageQuant Software (Molecular Dynamics, Sunnyvale, CA).

## EXAMPLE 5

Phosphoamino Acid Analysis

Samples subjected to *in vitro* kinase assays were separated by SDS-PAGE, transferred to PVDF membranes by electrophoresis, and stained with Ponceau S. Protein bands were excised, boiled with 6 N HCl for 1 hour, dried by speed vacuum, and analyzed by TLC in buffer containing 80% methanol, 1.5% acetic acid, and 0.5% formic acid. Subsequently, the dried TLC plate was analyzed by a Phosphorimager. Migration of standards was visualized by spraying the dried TLC plate with ninhydrin.

## EXAMPLE 6

Protein kinase associated with the p80 form of the TNF receptor

The present invention identifies proteins and protein kinase from U-937 cells that associate with the p80 cytoplasmic domain of the TNF receptor. Whether the cytoplasmic domain of p80 form of the tumor necrosis factor receptor could interact with proteins from either $^{35}$S-methionine/cysteine or $^{32}P_i$-labeled U-937 cells was first examined. Irrespective of TNF treatment, a protein was found to associate specifically with GST-p80CD in $^{35}$S-labeled cells (Figure 1, left). This protein, which had an approximate molecular mass of 58 $\pm$ 2 kDa, was detected in six separate studies.

To determine whether phosphoproteins could bind to GST-p80CD, cells were labeled with $^{32}P_i$. The present invention identified a phosphoprotein of approximately 60 $\pm$ 1 kDa (average

of four studies) that bound to GST-p80CD (Figure 1, right). In three of the four studies, the amount of phosphorylation of the associated phosphoprotein was increased when cells were treated with TNF. Thus, the protein identified by $^{35}$S-labeling was a phosphoprotein referred here on as pp59 and for the purposes of the present invention is termed p80TRAP as defined above.

A previous report indicated that overexpression of the p80 form of the human TNF receptor in the human embryonic kidney cell line 293 is constitutively phosphorylated on serine (97%) and threonine (3%) residues. Others have reported phosphorylation of the natural p80 TNF receptor on serine residues from SW480T cells. Although these reports show that the p80 receptor was phosphorylated *in vivo*, no kinase has been shown to be associated with this receptor. The present invention indicates that GST-p80CD serves as a substrate for several purified protein kinases.

## EXAMPLE 7

Phosphorylation of fusion protein

Employing the GST fusion protein system, the present invention shows that a protein kinase from U-937 cells associates with the cytoplasmic domain of the p80 form of the TNF receptor. As defined above, this kinase is termed p80TRAK for the purposes of the present invention. Figure 2 shows that GST-p80CD bound to glutathione-agarose adsorbed protein kinase from cell extracts that phosphorylated the fusion protein in a Mg-ATP-dependent manner. Under these conditions, neither kinase was found to be associated with GST alone nor was phosphorylated (Figure 2). A mock reaction devoid of cell extracts showed that the kinase activity originated from the cells and that the cytoplasmic domain had no intrinsic kinase activity. The fusion protein could be cleaved with thrombin (at a cleavage site between GST and p80CD) to show that only the cytoplasmic domain of p80 form of the TNF receptor was phosphorylated. Additionally, after *in vitro* kinase assays, a specific protein of approximately 59 kDa bound to GST-p80CD and was phosphorylated (Figure 2). Based on molecular size, this 59-kDa protein appears to be the same protein that was identified in both $^{35}$S- and $^{32}$P-labeled cells

(Figure 1). Furthermore, the cytoplasmic domain of the p60 receptor did not bind pp59 the protein (p80TRAP). The phosphorylation of a second smaller protein was most likely due to a degradation.

## EXAMPLE 8

### Phosphorylation of p80TRAP

The p80 form of the TNF receptor cytoplasmic domain-associated kinase activity was found to be optimal only in the presence of either $Mg^{++}$ or $Mn^{++}$, but not $Ca^{++}$ (Figure 3A). Phosphorylation of p80TRAP also preferred $Mn^{++}$ over $Mg^{++}$. Unlike reports that showed phosphorylation of p80 form of the TNF receptor primarily at serine, phosphoamino acid analysis in the present invention indicates that the phosphorylation of the p80 form of the TNF receptor occurred on serine and threonine residues (Figure 3B). Phosphorylation of p80TRAP, both *in vitro* and *in vivo*, was also found to occur on serine and threonine residues (Figure 7), suggesting that the p80 cytoplasmic domain-associated kinase phosphorylates p80TRAP *in vivo*.

Besides phosphorylating p80TRAP and the cytoplasmic domain of p80, the receptor-associated kinase (p80TRAK) also phosphorylated histone H2B and MBP (Figure 3C). Interestingly, the p80 receptor-associated kinase could also phosphorylate the cytoplasmic domain of the p60 form of the TNF receptor (Figure 3C). Thus, there exists a novel cross-talk between the two forms of the TNF receptor and that these two receptors bind distinct protein kinases.

## EXAMPLE 9

### Time-dependency of phosphorylation

It is known that the treatment with TNF of A293 cells overexpressing the p80 receptor yield no change in p80 receptor phosphorylation. In addition, pretreatment of SW480T cells with staurosporine reduced the extent of phosphorylation of the p80 receptor, suggesting, albeit indirectly, that a staurosporine-sensitive kinase(s) was being inhibited. The present invention demonstrates that TNF could induce the GST-p80CD-associated kinase activity from U-937 cells. Serum-starved U-937 cells treated with 5 nM TNF for various times were subjected to *in*

*vitro* binding assays with GST-p80CD, followed by kinase reactions with $Mg^{++}$ and MBP. TNF-dependent phosphorylation of MBP peaked between 15-25 minutes with approximately a 2-fold increase from time zero (Figure 4). This was observed in at least three separate experiments. Cells left untreated showed a basal level of p80 receptor-associated kinase activity with myelin basic protein as the substrate. In addition, both GST-p80CD and p80TRAP showed elevated levels of phosphorylation coincident with increased phosphorylation of myelin basic protein (Figure 4). In the present invention, the p80 receptor-associated kinase activity *in vitro* was measured in a time-dependent manner, whereas prior art experiments measured the phosphorylation of immunoprecipitated p80 receptor *in vivo* after 15 to 30 minutes treatment with TNF. Additionally, the p80 form of the tumor necrosis factor receptor may not be the optimum substrate for the associated kinase, as illustrated by the phosphorylation of myelin basic protein (Figure 4).

The cytoplasmic domain of p80 receptor comprises 174 amino acids and is shorter by 48 amino acids than the p60 cytoplasmic domain. Similar to the p60 receptor, the cytoplasmic domain of p80 contains a high content of proline, serine, threonine, and acidic residues; however, these are not clustered in regions as in the p60 receptor. Unlike the p60 receptor, the p80 receptor lacks tyrosine residues in its intracellular region.

## EXAMPLE 10

Purification of p80TRAK

Approximately 14.5 x $10^9$ U937 cells were grown to a density of 1.4 x $10^6$ cells/ml in 10.5 L of RPML-1640 medium in the presence of 10% FBS. Cells were collected by centrifugation, washed three times with 50 ml cold PBS, and lysed in approximately 120 ml lysis buffer for 30 minutes on ice. The lysate was cleared by centrifugation at 8000 rpm for 10 minutes at 4°C in a SS34 rotor. The supernatant was mixed with 1 mg of GST attached to glutathione-agarose beads overnight at 4°C with rocking. Next, the mixture was centrifuged at 3000 rpm for 10 minutes and the supernatant was mixed with 500 $\mu$l of a 50% slurry of GST-p80CD attached to glutathione-agarose beads for 4 hours

at 4°C. The mixture was centrifuged and equal volumes of the beads were transferred to 10 eppendorf tubes. The beads were washed by the following method and each wash was saved: six washes (600 μl each) of lysis buffer, five washes (600 μl each, 5 minutes rocking in between each wash) of 1 M NaCl in lysis buffer and five washes (600 μl each, 10 minutes rocking in between each wash) of 2 M NaCl, 10 mM EDTA in lysis buffer. Subsequently, each of the five tubes were washed onto a column with two washes (500 μl) and washed on the column with 1 ml of 2 M NaCl, 10 mM EDTA in lysis buffer followed by 1.5 ml lysis buffer. The 1 M and 2 M salt washes were concentrated and the buffer exchanges to a buffer consisting of 20 mM HEPES, 50 mM NaCl, 10% glycerol, 0.1% 2-mercaptoethanol, 10 mM NaF and 0.2 mM sodium orthovanadate. Kinase assays were performed with casein, MBP and GST-p80CD as substrates. Figure 5 shows that the cytoplasmic domain of the p80 (residues 266-439) was expressed in *E. coli* as a GST fusion protein and purified by affinity chromatography on glutathione agarose. The fusion protein has a molecular mass of approximately 54 kDa. Figure 6 shows that the p80TRAK was purified using an in vitro binding assay with GST-p80CD attached to glutathione-agarose as an affinity column as described above. The p80TRAK was found to elute from the affinity column in two elutions of 1 M and 2 M NaCl. The kinase activity was measured by using casein, MBP and GST-p80CD as substrates (Figure 6).

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred

embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

**WHAT IS CLAIMED IS:**

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: Aggarwal, Bharat B. and Darnay, Bryant G.

(ii) TITLE OF INVENTION: Tumor Necrosis Factor Receptor-I-Associated Proteins And Protein Kinase And Methods For Their Use

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:

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(C) CITY: Houston

(D) STATE: Texas

(E) COUNTRY: USA

(F) ZIP: 77056

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: DS, HD 1.44 Mb/1.44 Mo

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: WordPerfect 6.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Weiler, James F.

(B) REGISTRATION NUMBER: 16,040

(C) REFERENCE/DOCKET NUMBER: D-5718

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 713-626-8646

(B) TELEFAX: 713-963-5853

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:
(A) Description: other nucleic acid
(iii) HYPOTHETICAL: No
(iv) ANTISENSE: No
(vi) ORIGINAL SOURCE:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
CTAAGAGAAT TCGCTACCAA CGGTGGAAGT CC 32

(3) INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE:
(A) Description: other nucleic acid
(iii) HYPOTHETICAL: No
(iv) ANTISENSE: No
(vi) ORIGINAL SOURCE:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
GACGTACTCG AGTCATCTGA GAAGACT 27

(4) INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) Description: other nucleic acid

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vi) ORIGINAL SOURCE:

(B) STRAIN:

(C) INDIVIDUAL ISOLATE:

(D) DEVELOPMENTAL STAGE:

(F) TISSUE TYPE:

(G) CELL TYPE:

(H) CELL LINE:

(ix) FEATURE:

(A) OTHER:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTAAGAGGAT CCAAAAAGAA GCCCTTGTGC CTG 33

(5) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) Description: other nucleic acid

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vi) ORIGINAL SOURCE:

(B) STRAIN:

(C) INDIVIDUAL ISOLATE:

(D) DEVELOPMENTAL STAGE:

(F) TISSUE TYPE:

(G) CELL TYPE:

(H) CELL LINE:

(ix) FEATURE:

(A) OTHER:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GACGTACTCG AGTCATCTGA GAAGACT 27

## Claims

1. An isolated and purified protein that associates with the cytoplasmic domain of the p80 form of the tumor necrosis factor receptor, having a molecular weight of 59 kDa as determined by SDS-PAGE, is a phosphoprotein, and does not bind to the p60 form of the tumor necrosis factor receptor.

2. The protein of claim 1, wherein said phosphoprotein is phosphorylated at serine and threonine residues.

3. The protein of claim 1, wherein said protein exhibits optimal phosphorylation in the presence of Mn††.

4. An isolated and purified protein kinase that associates with the cytoplasmic domain of the p80 form of the tumor necrosis factor receptor, said kinase phosphorylates both the p80 and p60 forms of the tumor necrosis factor receptor and phosphorylates a 59 kDa molecular weight protein associated with the cytoplasmic domain of the p80 form of the tumor necrosis factor receptor.

5. A pharmaceutical composition comprising an competitive inhibitor of the kinase of claim 4 and a pharmaceutically acceptable carrier.

6. A method of decreasing the biological effects of tumor necrosis factor comprising the step of inhibiting the phosphorylation of the protein of claim 1.

7. The method of claim 6, wherein said protein is inhibited by administering a pharmacologically effective dose of the pharmaceutical composition of claim 5.

8. A method of decreasing the biological effects of tumor necrosis factor comprising the step of inhibiting the kinase of claim 4.

9. A method of reducing the cytotoxic effects of tumor necrosis factor comprising the step of inhibiting the kinase of claim 4.

10. A method of treating a pathophysiological state characterized by an undesirable physiological level of tumor necrosis factor comprising the step of administering a pharmacologically effective dose of the pharmaceutical composition of claim 5 to a human.

11. The method of claim 10, wherein the pathophysiological state is selected from the group consisting of neoplastic disease, human immunodeficiency disease, sepsis, cachexia, graft vs host disease, autoimmune disease, cerebral malaria and capillary leak syndrome.

12. The method of claim 11, wherein said neoplastic disease is selected from the group consisting of leukemia, ovarian carcinoma, renal cell carcinoma, breast adenocarcinoma and glioblastoma.

13. The method of claim 11, wherein said autoimmune disease is selected from the group consisting of systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis.

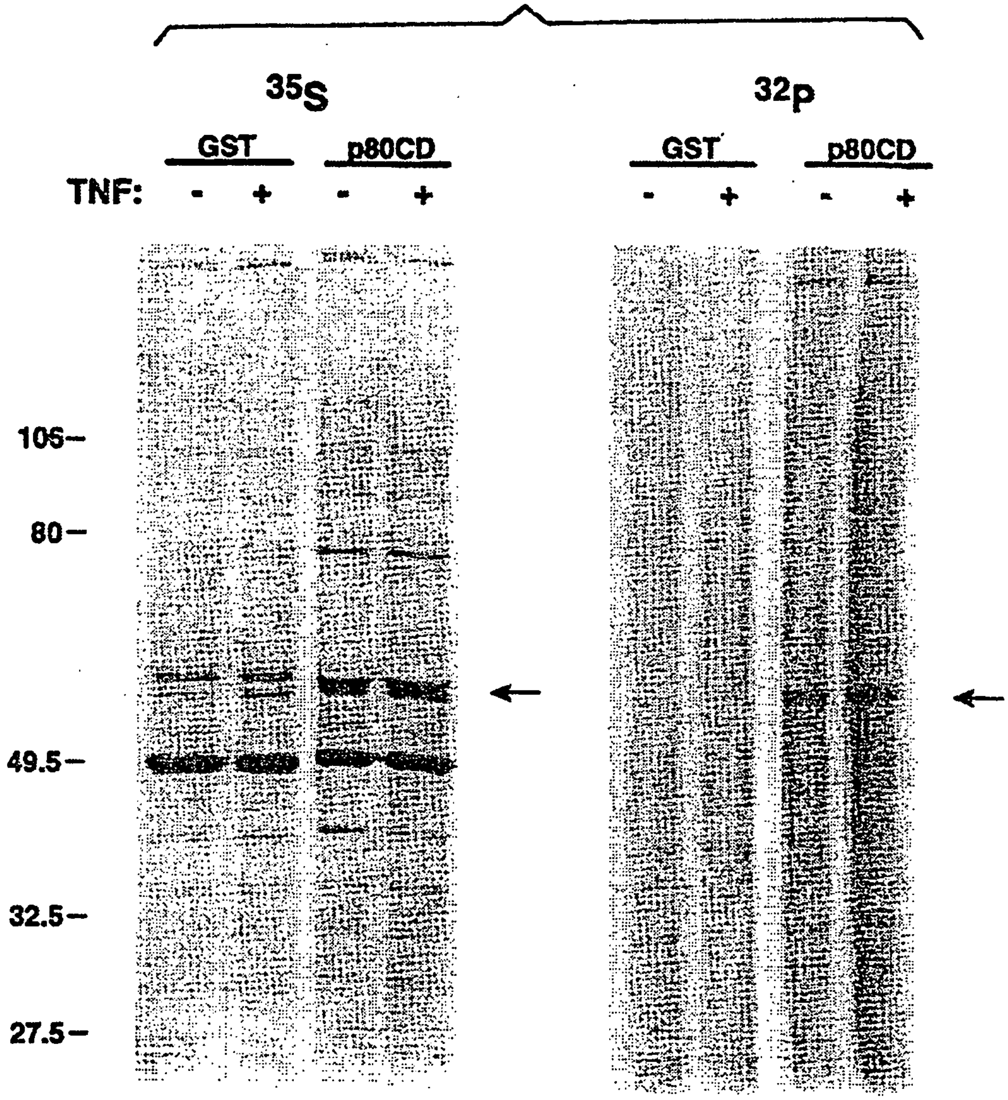



FIG. 1

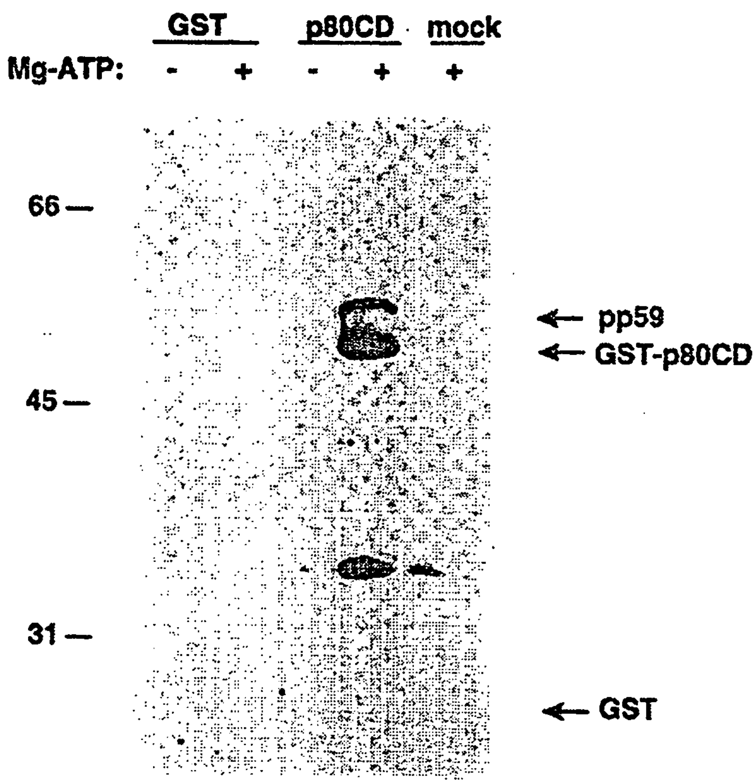
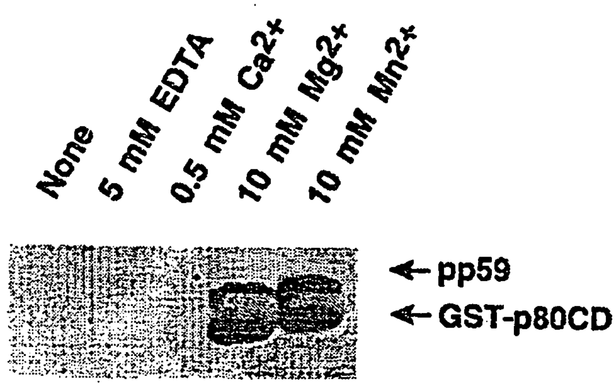



FIG. 2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0.5 mM Ca2+
10 mM Mg2+
10 mM Mn2+
pp59
GST-p80CD

FIG. 3A

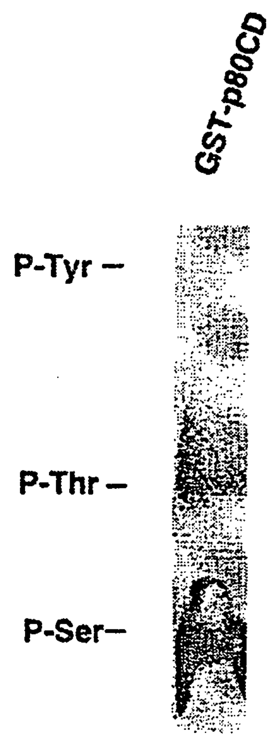
GST-p80CD
P-Tyr
P-Thr
P-Ser

FIG. 3B

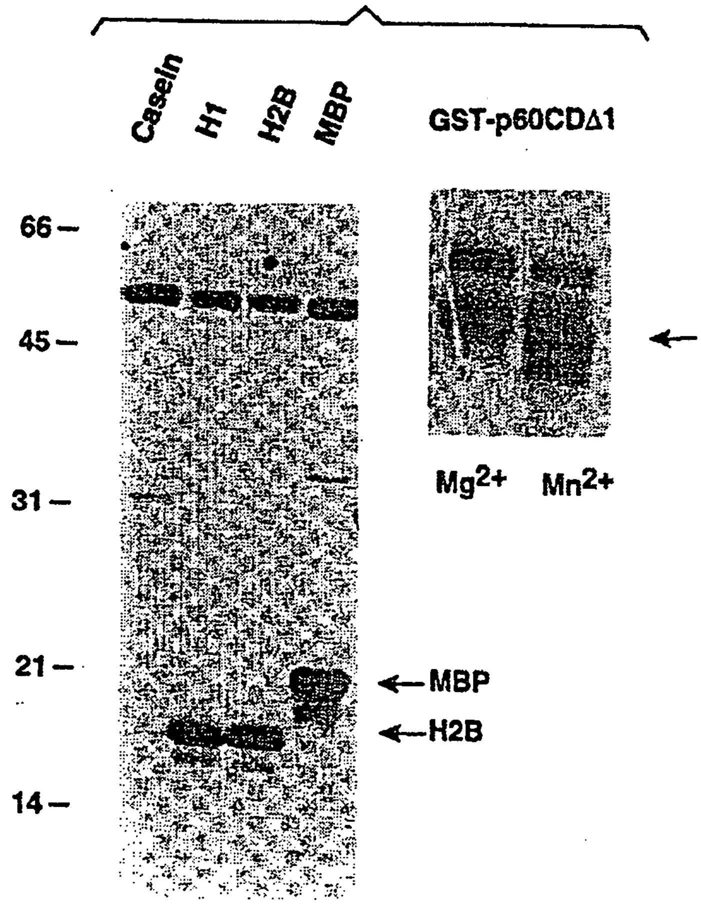


FIG. 3C

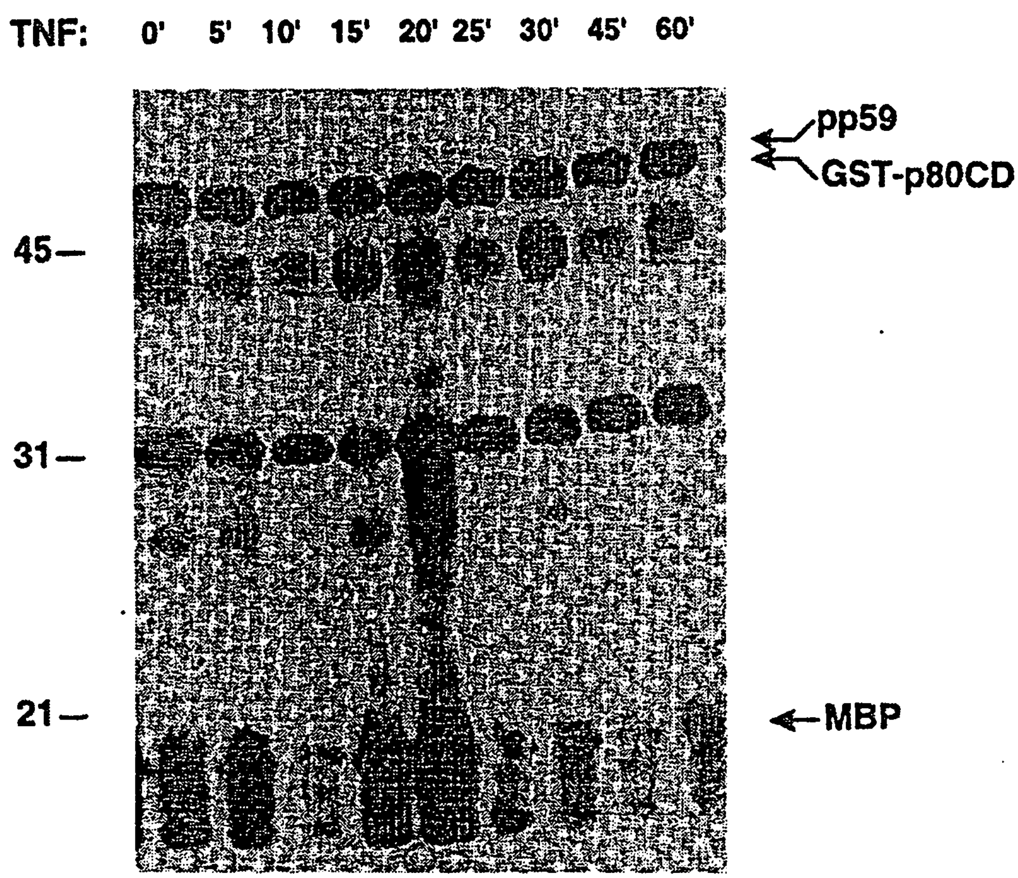


FIG. 4

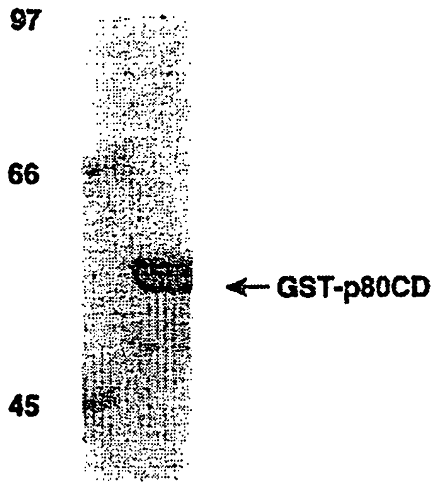


FIG. 5

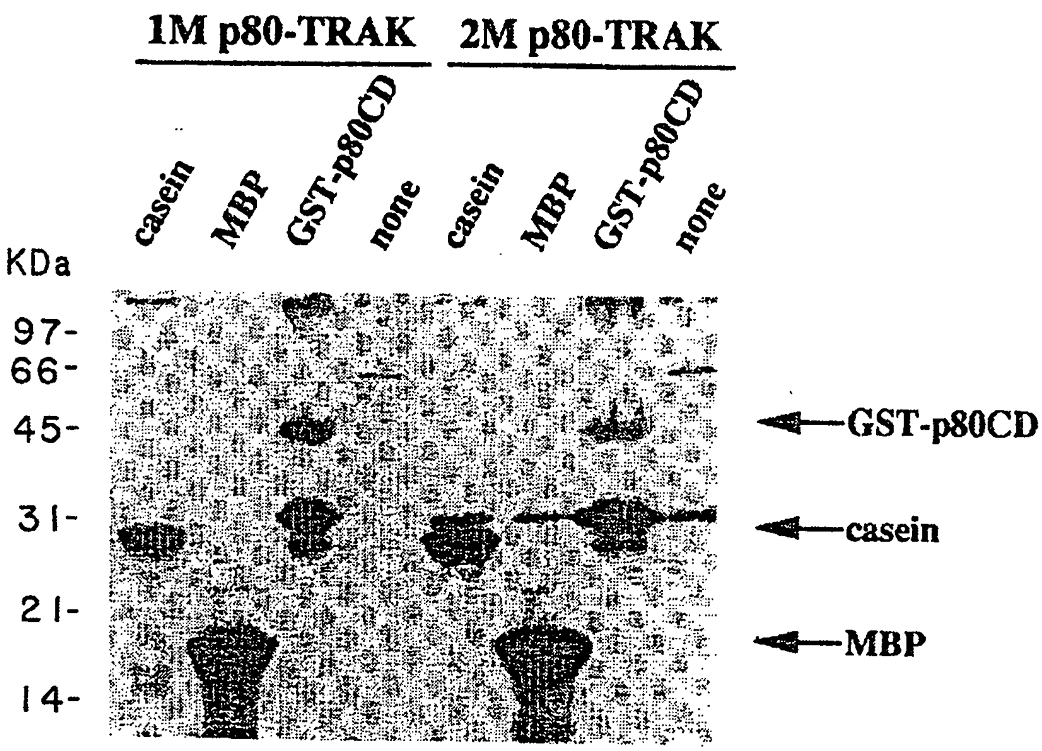
1M p80-TRAK
2M p80-TRAK
casein
MBP
GST-p80CD
none
casein
MBP
GST-p80CD
none
KDa
97-
66-
45-
31-
21-
14-
GST-p80CD
casein
MBP

FIG. 6

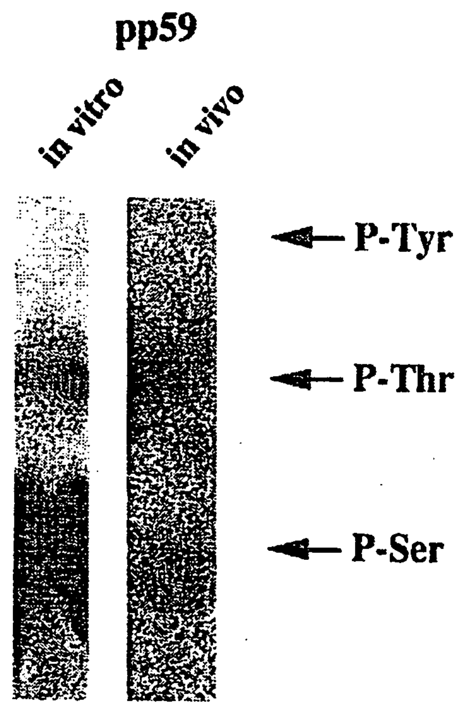


FIG. 7

## INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/08273

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 38/00, 38/16; C07K 14/00; C12N 09/12
US CL :435/194; 514/7, 12, 21; 530/352
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/194; 514/7, 12, 21; 530/352

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, WPIDS, SCISEARCH, BIOSIS, EMBASE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A, P | J. Immunol., Volume 153, Number 8, issued 15 October, 1994, Zhang et al, "Role of Sulfhydryl Groups in Induction of Cell Surface Down-Modulation and Shedding of Extracellular Domain of Human TNF Receptors in Human Histiolytic Lymphoma U937 Cells", pages 3745-3754, see entire document. | 1-13 |
| A | J. Biol. Chem., Volume 269, Number 20, issued 20 May 1994, Chaturvedi et al, "Tumor Necrosis Factor and Lymphotoxin", pages 14575-14583, see entire document. | 1-13 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

26 SEPTEMBER 1995

Date of mailing of the international search report

13 OCT 1995

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## INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/08273

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | J. Immunol., Volume 152, Number 8, issued 15 April 1994, Higuchi et al, "Differential Roles of Two Types of the TNF Receptor in TNF-Induced Cytotoxicity, DNA Fragmentation, and Differentiation", pages 4017-4025, see the entire document. | 1-13 |
| A, P | J. Biol. Chem., Volume 269, Number 32, issued 12 August 1994, Darnay et al, "Identification of a Protein Kinase Assiciated with the Cytoplasmic Domain of the p60 Tumor Necrosis Factor Receptor", pages 20299-20304, see the entire document. | 4-13 |